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PITUITARY FOLLICULAR CELLS SECRETE BOTH VASCULAR ENDOTHELIAL GROWTH FACTOR AND FOLLISTATIN

*D. Gospodarowicz and #K. Lau

*Cancer Research Institute, University of California Medical Center, San Francisco, CA 94143

#California Biotechnology Inc., 2450 Bayshore Parkway, MountainView, CA 94043

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Follistatin, a hormone which acts to suppress the release of follicle-stimulating hormone (FSH) by pituitary-derived gonadotrophs, has previously been identified only in the liquor folliculi of ovarian follicles. By microsequencing of fractions derived from conditioned medium, we show here that bovine pituitary-derived folliculo stellate cells are also capable of producing and secreting this hormone. These results suggest that folliculo stellate cells may serve as a source of follistatin within the pituitary itself and that the regulation of FSH release from the pituitary could therefore involve a paracrine mechanism.

We have previously reported that pituitary-derived folliculo stellate or follicular (FS) cells secrete a mitogen that appears to be specific for vascular endothelial cells. This mitogen has provisionally been named either folliculo stellate-derived growth factor (FSdGF; ref. 1) or vascular endothelial growth factor (VEGF; ref. 2). Two methods have been reported for the isolation of this mitogen from the FS cell conditioned medium: one, involving ammonium sulfate precipitation, heparin-Sepharose affinity chromatography, Bio Gel P-60 exclusion chromatography, Mono-S ion-exchange chromatography, and C4 reversed phase HPLC (1); the other, involving ammonium sulfate precipitation followed by fractionation over heparin-Sepharose and two C4 reversed phase HPLC steps (2). In isolating VEGF from FS cell conditioned medium using variations of the second purification scheme, we obtained fractions that consisted of two major components. We report here that microsequencing of these fractions demonstrated that the two components were VEGF and, unexpectedly, follistatin, a hormone which acts to regulate the release of follicle stimulating hormone (FSH) from the pituitary (3,4).

MATERIALS AND METHODS

Materials Tissue culture reagents were obtained from Gibco (Grand Island, N.Y.) through the UCSF Cell Culture Facility. Tissue culture plates, acetonitrile, 2-propanol, heparin-Sepharose (H-S), Vydac C4 HPLC columns, molecular weight markers, and protein determination kits were obtained as described in ref. 2.

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Collection of conditioned medium from FS cell cultures Confluent cultures of bovine pituitary FS cells were established in large scale Nunc tissue culture plates, as previously described (2). After several washes with PBS to remove the serum-containing growth medium, the confluent monolayers were incubated in a serum-free medium consisting of low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with transferrin (10 µg/ml), insulin (5 µg/ml), leupeptin (5 µg/ml), glutamine (2 mM), gentamicin (50µg/ml) and Fungizone (2.5 µg/ml). Conditioned medium (CM) was collected and replaced with fresh medium every two to three days.

Concentration of conditioned medium Floating cells and cell debris were removed by centrifugation (10,000 x g, 15 min., 4°C) from the freshly collected CM. The pH of the supernatant was then adjusted to 5.6 with 6 N HCl. Ammonium sulfate (520 g/liter) was added, and the suspension was set for 6 hr at 4°C; the precipitate was then collected by centrifugation (10,000 x g, 30 min, 4°C), redissolved in PBS to 1% of the original volume of the CM, and stored at -70°C.

Fractionation procedures The concentrated CM was thawed and dialyzed against 0.01 M Tris-HCl, pH 7.2, 0.05 M NaCl (12 hrs, 4°C). The dialysate was applied to an H-S column (10 ml) pre-equilibrated with 0.01 M Tris-HCl, pH 7.2, 0.05 M NaCl. The pre-equilibration buffer was used to wash the column until the absorbance at 280 nm had returned to baseline. The column was then eluted stepwise with 0.01 M Tris-HCl, pH 7.2, containing 0.15M, 0.9 M, and 3M NaCl, as described in ref 2. Alternatively, the column was washed with 0.01 M Tris-HCl, pH 7.2, containing 0.15, 0.45, and 1 M NaCl, as described in ref. 1. The flow rate was 1.5 ml/min, and fractions of 1.5 ml were collected.

Bioactive fractions from the H-S chromatography (0.9 M or 1 M NaCl pool) were diluted four-fold with 0.1% trifluoroacetic acid (TFA) in water, applied to a semi-preparative Vydac C_4 HPLC column (10 x 250 mm) equilibrated in 0.1% TFA/20% acetonitrile, and eluted with a linear gradient of acetonitrile (20 - 45% in 117 min) as described (2). The flow rate was 2 ml/min, and 3 ml fractions were collected. Fractions containing the peak of bioactivity were pooled and diluted two-fold in 0.1% TFA/20% propanol, and applied to an analytical C_4 column (4.6 x 250 mm) equilibrated with 0.1% TFA/20% propanol. The column was eluted with a linear gradient of 2-propanol (20 - 45% in 117 min). The flow rate was 0.6 ml/min, and 1.5 ml fractions were collected, as described (2). After removal of aliquots for bioassay and protein microsequencing, the bioactive fractions were dried in a Speed-Vac before analysis by polyacrylamide gel electrophoresis (PAGE) (5). Microsequencing of the most bioactive fractions was carried out by direct application of fraction aliquots to protein sequenator Model 470A or 477A (Applied Biosystems). Edman degradation cycles were carried out and identifications of amino acid derivatives were made by an on-line HPLC column.

Bioassays Bovine adrenal cortex derived capillary endothelial (ACE) cells were maintained as previously described (6). For bioassay, cells were seeded at a density of 1 x 10^4 cells/well in 1 ml of medium in 12-multiwell plates. Aliquots of fractions to be assayed were diluted in PBS containing 0.2% gelatin, and were then added to the cells at 5 µl/ml of assay medium. After four or five days, cells were trypsinized and cell densities were determined in a Coulter counter.

RESULTS

Two independent fractionations of FS cell conditioned medium were carried out. Both of these fractionation schemes involved ammonium sulfate precipitation followed by H-S chromatography, reversed phase HPLC with an acetonitrile gradient elution, and finally, reversed phase HPLC with a 2-propanol gradient elution (2). In the first scheme, the steps were performed essentially as described by Ferrara and

Henzel (2); in the second scheme, the heparin column elution followed the method described in ref. 1.

The results obtained for the H-S chromatography step in each of the two fractionation schemes are shown in Fig. 1. In both cases, material not retained by the column was inactive and accounted for approximately 50% of the total protein loaded. In the first fractionation run (Fig. 1A), the H-S column was eluted stepwise with 0.15 M, 0.9 M, and then 3 M NaCl, as described (2). Elution with 0.15 M NaCl yielded a small peak of protein with no bioactivity, while elution with 0.9 M NaCl yielded a major peak of protein with approximately 90% of the bioactivity applied to the column (Fig. 1A). Protein recovery in the pooled 0.9 M NaCl fractions was 32% of the total protein loaded on the column. Assuming that the recovery of biological activity is 100%, this step provided a 3- fold overall purification.

Since the elution conditions used in the experiment shown in Fig. 1A did not provide as significant a purification of the starting material as we had previously observed using this type of column (1), we subjected a second batch of concentrated CM to H-S column chromatography, and used the elution conditions described in ref.1 (0.15 M, 0.45 M, and 1 M NaCl elution steps; Fig. 1B). Most of the retained proteins were eluted with 0.45 M NaCl, while 90% of the bioactivity was present along with 3%

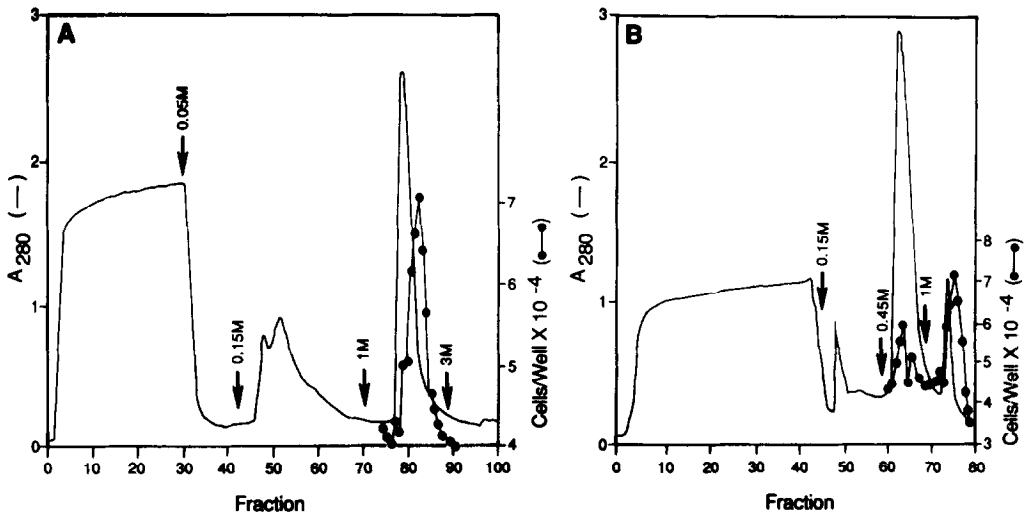

Fig. 1. Heparin-Sepharose fractionations of FS cell conditioned medium. A. CM (6 liters) concentrated by ammonium sulfate precipitation was applied to an H-S column equilibrated with 0.01 M Tris-HCl, pH 7.2, 0.05 M NaCl. After extensive washing with equilibration buffer, the column was eluted sequentially with 0.01 M Tris-HCl, pH 7.2, containing 0.15 M, 0.9 M, and 3 M NaCl (ref. 2). Aliquots of the fractions were diluted 100-fold in PBS containing 0.2% gelatin for bioassay on endothelial cells. B. CM (12 liters) was concentrated and fractionated on an H-S column as described above, except that the stepwise elutions were performed with 0.01 M Tris-HCl, pH 7.2, containing 0.15 M, 0.45 M, and 1 M NaCl (ref. 1).

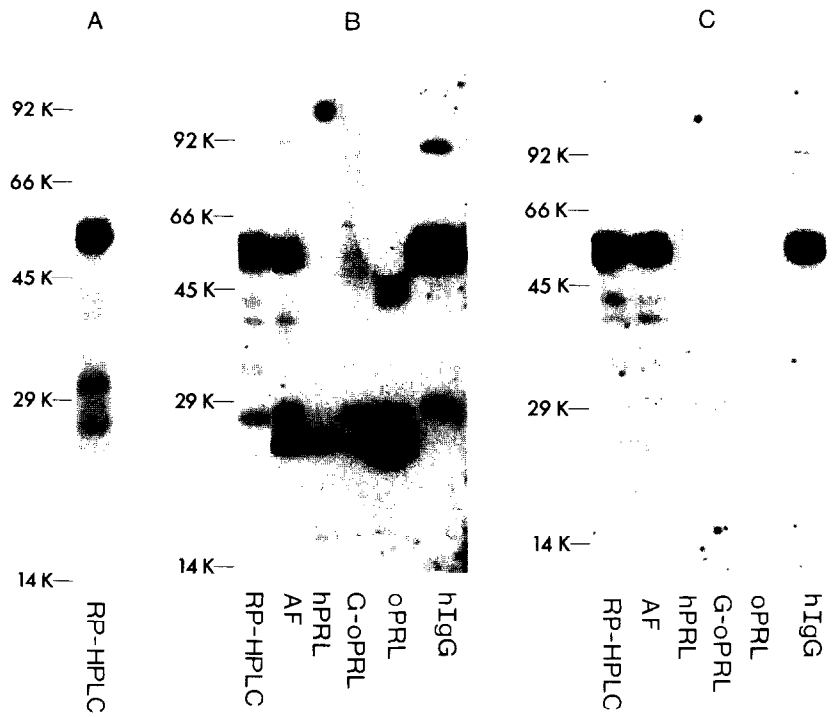

FIGURE 1
Silver stained gel and two Western blots demonstrating the association of immunospecific anti-hPRL binding to the 25 kDa protein thought to be G-PRL and the nonspecific binding of protein A to the 51 kDa band thought to be immunoglobulin heavy chains.
Amniotic fluid was subjected to ultrafiltration against a 50 kDa exclusion filter, chromatographed using DEAE-HPLC in the presence of 6M urea and the unbound fractions subjected to reverse phase - HPLC. Each of the six peaks eluted from the RP-HPLC column were then concentrated and electrophoresed on a 12% polyacrylamide gel. Only peak VI, which eluted at 45-55% acetonitrile, contained immunoreactive PRL.
A. Silver stain of RP-HPLC peak VI. B. Western blot of RP-HPLC peak VI and standards using rabbit anti-PRL serum and radioactive detection with $[^{125}I]$protein A. C. Western blot of same, using nonimmune rabbit serum and radioactive detection with $[^{125}I]$protein A. hIgG = 6 μg human IgG (Sigma); oPRL = 3 μg ovine PRL (Sigma); G-PRL = 500 ng ovine G-PRL; hPRL = 60 ng NIADDK hPRL I-7; AF = 50 μl amniotic fluid; RP-HPLC = aliquot of eluant in peak VI from reverse phase HPLC, equivalent to 500 μl starting volume of amniotic fluid.

Immunoblotting showed that G-PRL moved into this region of the chromatogram, which corresponds to the position of purified G-PRL. No such movement was observed in the absence of 2-mercaptoethanol. Similar results were obtained with SDS-PAGE and gel filtration HPLC of amniotic fluid. In the absence of 2-mercaptoethanol, the G-PRL was associated with a large complex that contained immunoglobulin; in the presence of a reducing agent, the G-PRL and immunoglobulin separated. Substantially fewer immunoglobulin breakdown products were found in these blots, presumably because of the absence of prior urea treatment.

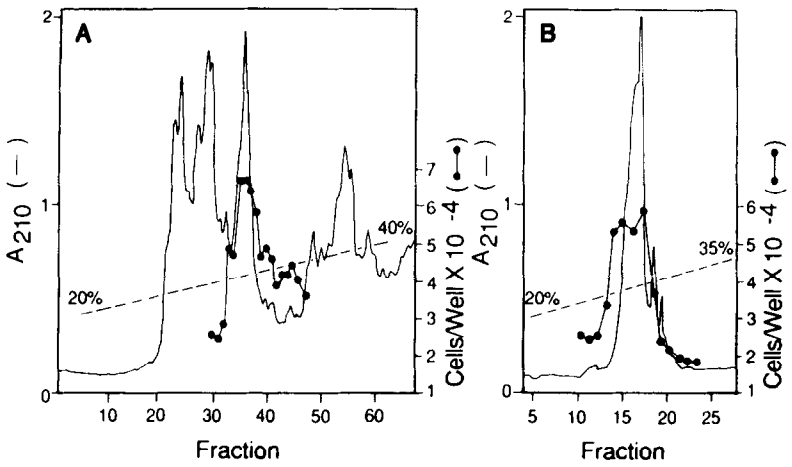

Fig. 3. Reversed phase HPLC fractionation of mitogenic activity partially purified by the second H-S column. A. The peak bioactive fractions from the elution shown in Fig. 1B (fractions 72 to 78) were chromatographed over a semi-preparative (panel A) and then an analytical (panel B) C_4 reversed phase HPLC column, as described in the legend to Fig. 2.

peak in the range of 29-31% acetonitrile. Half of the content of the pooled bioactive fractions was then chromatographed on the second, analytical C_4 reversed phase HPLC column, using a gradient of 2-propanol for column elution (Fig. 3B). A single peak of bioactive material was again obtained, corresponding to an asymmetrical peak of UV absorbing material with multiple shoulders. The pooled bioactive fractions from the elution shown in Fig. 3B had an ED_{50} of 3 ng/ml when assayed on ACE cells.

When analyzed by SDS-PAGE, most of the fractions of peak bioactivity in the elution shown in Fig. 3B displayed multiple components (Fig. 4B). The mixed nature of the fractions was particularly evident after reduction of the samples. One of the bands in the reduced fractions had a Mr of 23 kD, which corresponds to the apparent Mr of the monomeric form of VEGF (1,2). The 23 kD band had a staining intensity which was no more than 5 to 10% of the total load of protein in each of the fractions, except fraction 14, which appeared to be mostly the 23 kD species. Microsequencing of fraction 14 yielded a nearly homogeneous sequence that indeed corresponds to the known N-terminal sequence of VEGF (APMAEGGQKPXE; ref.1). Microsequencing of the other three fractions yielded a mixture of amino acids in each sequencing cycle. In the case of fraction 17, the mixture contained two distinct major sequences, one of which corresponds to VEGF (APMAEGGQKPH). Subtracting out this sequence from the major dual sequence obtained left the sequence GNXWLRQAKNG, which corresponds exactly to the known N-terminal sequence of porcine and bovine follistatin (GNCWLRQAKNG; refs. 3,4). This single-chain protein has been isolated in three molecular weight forms from bovine follicular fluid (31 kD, 35 kD, and 39 kD; ref. 4),

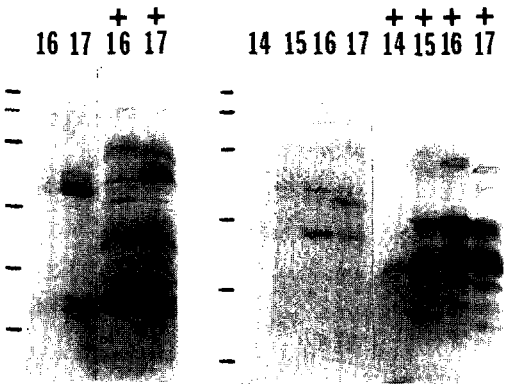

Fig. 4. SDS-PAGE analysis of peak bioactive fractions from analytical C4 HPLC elutions. A. 250 µl aliquots of fractions 16 and 17 (Fig. 2B) were dried and redissolved in sample buffer with (+) or without 0.01 M dithiothreitol. After heat denaturation, the samples were electrophoresed in a 15% SDS-PAGE gel which was subsequently silver stained. B. 250 µl aliquots of fractions 14 through 17 (Fig. 3B) were dried and analyzed by SDS-PAGE as described above. Molecular weight markers are: phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin 43,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400.

and bands of approximately these molecular weights are present in the SDS-PAGE gel analysis of fraction 17 (Fig. 4B).

The peak bioactive fractions from the HPLC elution shown in Fig. 2B were also analyzed by SDS-PAGE, and again showed multiple bands under both reduced and nonreduced conditions (Fig. 4A). A 23 kD band, corresponding to approximately 10% in staining intensity of the total protein load, is apparent in both fraction 16 and 17 after electrophoresis under reducing conditions, as are bands in the 31-39 kD range. Microsequencing of the pooled fractions 16 and 17 yielded a mixture of amino acids at each degradation cycle, with no major sequences standing out. The mixed sequence did contain, however, the amino acids predicted if the fractions analyzed contained both VEGF and follistatin. Thus, using sequential chromatography over H-S, C4 HPLC eluted with acetonitrile, and C4 HPLC eluted with 2-propanol (2), we found that in both fractionations of FS cell conditioned medium VEGF essentiallly co-eluted with follistatin.

DISCUSSION

Using the fast and simple VEGF purification scheme developed by Ferrara and Henzel (2) we have not been able to purify VEGF to homogeneity. This is in agreement with a previous study (1), which led us to adopt a more complex purification protocol, including a step of gel exclusion chromatography to eliminate low molecular weight contaminants (Mr 14 to 30 kD) and a step of ion exchange chromatography to eliminate contaminants with Mr of 32 to 38 kD, similar to those reported for follistatin (3,4). Previous studies (4,7) have shown that on H-S and C4

reversed phase columns, the chromatographic behaviour of follistatin is very close, if not identical, to that of VEGF. It is therefore not surprising to find follistatin as a major contaminant in the final VEGF preparation using the purification schemes employed here.

The previous identification of follistatin in ovarian liquor folliculi (4,7) does not have an obvious physiological meaning at the pituitary level. In contrast, follistatin production by FS cells implies that they could be involved in the paracrine control of FSH release by gonadotrophs. Based on indirect evidences, it has already been proposed that FS cells could be involved in the modulation of pituitary hormone release (8). Two obvious questions are raised by our present finding: do FS cells produce any known or novel modulators of the release of other pituitary hormones such as MSH, GH, PRL, and LH, and, is the release of follistatin and other putative mediators by FS cells under the control of neurosecretory factors?

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